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Changes in Hepatitis C Virus Quasispecies and Density Populations in Patients Before and After Interferon Therapy

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Some chronic hepatitis C patients show sustained response to interferon (IFN) therapy despite viremia. This condition seems to be related to the density populations of hepatitis C virus (HCV) [Kanto et al. (1995): J Med Virol 46:230–237]. To investigate further the relationship between alanine aminotransferase (ALT) levels after IFN therapy and the HCV density populations, we undertook differential flotation centrifugation of HCV and single strand conformation polymorphism targeted the hypervariable region (HVR) of E2 glycoprotein, which seems to be related to the density populations. Sera were obtained serially from 12 patients who had undergone IFN therapy (six sustained responders with viremia, six nonresponders). During the follow-up after interferon therapy, the HVR heterogeneities changed in 9 of the 12 patients. The remaining three patients whose heterogeneities did not changed persistently showed normal ALT. The changes in HVR heterogeneities were less pronounced in the sustained responders with viremia than in nonresponders; however, their density populations were prominently high in both responders. In two cases, changes in HVR heterogeneities and increase in low-density virion were observed before the hepatitis flare-up. These data indicate that HVR quasispecies show more relation to ALT levels after IFN therapy than HCV density populations and that the changes in the HVR sequences and HCV density populations may be associated with ALT elevation in some patients.

KEY WORDS: HCV, differential flotation centrifugation, single strand conformation polymorphism

INTRODUCTION

Interferon (IFN) is the most effective drug available for treating hepatitis C virus (HCV) infections. A majority of the patients who persistently show return to normal of alanine aminotransferase (ALT) levels after IFN therapy also show disappearance of HCV from the serum. However, some patients have viremia in spite of persistently normal ALT levels after IFN therapy [Lau et al., 1993]. Kanto et al. [1995] suggested that the HCV density population is closely related to the ALT levels after IFN therapy. HCV appears to circulate in various forms, such as native virion, immune complexes, and nucleocapsids, during infections. This fact has been determined by equilibrium centrifugation, differential flotation centrifugation, and immunoprecipitation [Miyamoto et al., 1992; Kanto et al., 1995; Hijikata et al., 1993]. The immune complexes and/or nucleocapsid forms revealed high-density virion, whereas the native form revealed low-density virion [Hijikata et al., 1993].

Significant genetic heterogeneities have been reported for HCVs obtained from different geographical regions [Kato et al., 1990a,b; Okamoto et al., 1992a] and for those isolated from sera taken from single individuals and chimpanzees over a long period of time [Okamoto et al., 1992a, Ogata et al., 1991; Weiner et al., 1992]. The heterogeneity is distributed unevenly in the virus genome, particularly in the gene encoding the 5′ end of the envelope protein-coding region (E2/NS1) called hypervariable region-1 (HVR-1) [Hijikata et al., 1991]. The HVR-1 seems to contain the epitope for neutralizing antibodies, and the hypervariability of this region is reported to be due to mutations that lead to persistent HCV infection [Weiner et al., 1992; Taniguchi et al., 1993; Kato et al., 1993; Kojima et al., 1994]. This variation, which is found in infected individuals, is called quasispecies. The quasispecies should therefore be modified by immune pressure, interferon (IFN) [Weiner et al., 1992; Higashi et al., 1993], and various environmental factors such as liver cell damage [Kurosaki et al., 1994]. So far, little is known about its significance in terms of the clinical features.

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Because HCV is an enveloped virus, the immune complex of HCV in circulation is in the form of antienvelope antibodies, located particularly in the HVR-1. It is, suggested therefore, that HVR-1 quasispecies are closely related to various circulating forms of HCV.

In the present study, we investigated the HVR-1 quasispecies and the HCV density populations of sera sequentially obtained from HCV-infected patients treated with IFN and determined how these aspects related to the ALT levels after IFN therapy. The HVR-1 quasispecies were analyzed by single-strand conformation polymorphism (SSCP) [Yap and McGee, 1992], and the HCV density populations were studied by differential flotation centrifugation [Hijikata et al., 1993]. The results suggested that IFN accelerated the mutation in HVR and affected the density populations of HCV and that HVR-1 quasispecies were associated with liver dysfunction, which had resulted possibly from cytotoxic immunity.

PATIENTS AND METHODS

Patients

Twelve patients with chronic hepatitis C were studied. Diagnosis was based on anti-HCV antibody (EIA2; Abbott Laboratories, North Chicago, IL), serum HCV RNA, and liver histology. The patients underwent IFN therapy but failed to respond virologically. They were divided into two groups according to their ALT patterns after IFN therapy. One group consisted of patients having continuously normal ALT, though with viremia, for at least 6 months after IFN therapy (incomplete response; IR); the other group consisted of patients who showed abnormal ALT within 6 months after the cessation of IFN therapy (nonresponse; NR). Six of the twelve patients were in the IR group and the remaining six in the NR group. The clinical characteristics of the patients were summarized in Table I.

TABLE I. Patient Characteristics and Vial Marker*

Patient	Sex	Age	Effect	Genotype	Histology	IFN[a]
1	M	60	IR	1b	CAH2B	468
2	M	63	IR	1b	CAH2A	528
3	M	53	IR	2b	CAH2A	528
4	F	29	IR	1b	CAH2A	76.5
5	M	66	IR	1b	CAH2A	528
6	F	67	IR	1b	CAH2A	400
7	M	57	NR	2a	CAH2A	198
8	M	50	NR	1b	CAH2B	432
9	M	39	NR	1b	CAH2A	480
10	F	56	NR	1b	CAH2A	468
11	F	59	NR	1b	CAH2B	180
12	F	30	NR	1b	CAH2A	336

*Effect, response to IFN therapy; IR, incomplete response; NR, nonresponse.
[a]Data are expressed as total dose (megaunits).

RNA Extraction and Complementary DNA Synthesis

The HCV RNA was extracted from 100 μl of serum using a commercial kit (Sepa Gene-RV; Sanko Junyaku Co., Ltd, Tokyo, Japan). The HCV RNA pellets were then dissolved in 10 μl of distilled water and mixed with Molony murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD), RNase inhibitor (Promega, Madison, WI), and random primer (Takara Biomedicals, Kyoto, Japan) to a final volume of 20 μl. The HCV RNA solution was incubated for 1 hour at 37°C to convert it to complementary DNA (cDNA).

Polymerase Chain Reaction for SSCP

The HCV cDNA was amplified by the polymerase chain reaction (first-round PCR) using the following procedures; 2.5 μl of the cDNA was added to the reaction mixture (10 mmol/liter Tris-HCl, pH 8.3, 2.5 mmol/liter $MgCl_2$, 50 mmol/liter KCl, 200 μmol/liter deoxynucleotide triphosphate), 5 pmol each of the sense- and antisense-strand primers, and 1 unit of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) to a final volume of 25 μl. In general, each reaction cycle was carried out at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. There were 40 reaction cycles in total. The sense primer used was 5′-TGGCTTGGGACATGATGATGAAC-3′ [nucleotides (nt) 1285–1307 of HCV-BK (Takamizawa et al., 1991)]. The antisense primers used were 5′-GGGGTGAAGCAATACACTGGACCACA-3′ [nt 1277–1299 of HCV-J4 (Okamoto et al., 1990)] and 5′-GGGGTGAAGCAGTACACTGGGCCGCA-3′ (nt 1839–1864 of HCV-BK). The second PCR was carried out under the same conditions, except for 30 cycles for the first-round PCR as follows; 1 μl of the first PCR product was mixed to the same parameters. The sense primer used was 5′-GCCTTGCCTACTATTCCATG-3′ (nt 1405–1424 of HCV-BK) or 5′-GCTTAGCCTACTTCTCTATG-3′ [nt 1413–1424 of HCV-J6 (Okamoto et al., 1991)]. The antisense primer used was 5′-TTGATGTGCCAACTGCCATT-3′ (nt 1581–1600 of HCV-BK). The final PCR products were 196 bp in length including the HVR-1.

PCR-SSCP Analysis

SSCP analysis was carried out as described previously, with minor modifications [Yap and McGee, 1992]. Eight microliters of the second PCR product was mixed with 4 μl of stop solution and then denatured at 94°C for 3 minutes. The denatured product was chilled on ice for 10 minutes and then electrophoresed in a modified nondenaturing acrylamide gel (MDE gel; AT Biochem Inc., Malvern, PA) at room temperature using 100 V for 4 hours. The PCR product was stained with ethidium bromide and was observed under UV light.

Sensitivity of SSCP Analysis

To examine the sensitivity of SSCP, we selected two samples of single SSCP band that had different SCP mobilities (lanes 1 and 2 in Fig. 1). The first PCR products of the two were treated with Suprec-02 to remove the residual primers and dNTP, and then their DNA concentrations were measured with a DU Series 60 Spectrophotometer (Beckman Instruments, Inc.). The treated PCR products were then diluted to 8 ng/μl. Five microliters each of a 2^{-1}:2^{-5} dilution of 8 ng/μl one PCR

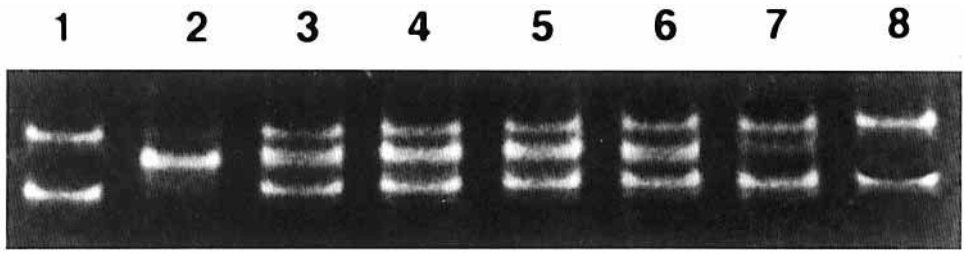

Fig. 1. Sensitivity of the SSCP analysis (see Materials and Methods). **Lanes 1** and **2** show the results of SSCP analysis in HVR using sera obtained from different patients. The first PCR products of these two samples were treated with SUPREC-02. One DNA sample (from lane 1) was made up to 8 ng/μl, and the other DNA sample (from lane 2) was diluted from 8 to 0.25 ng/μl (serial twofold increment). One microliter of each dilution was mixed, and the mixture was applied to the second PCR. **Lane 3:** 8 ng:8 ng; **lane 4:** 8 ng:4 ng; **lane 5:** 8 ng:2 ng; **lane 6:** 8 ng:1 ng; **lane 7:** 8 ng:0.5 ng; **lane 8:** 8 ng:0.25 ng.

product (from lane 2 in Fig. 1) was mixed with an equal volume of undiluted 8 ng/μl of the other PCR product (from lane in Fig. 1). The second PCR was carried out on 1 μl of these mixtures, and SSCP analysis was performed to determine the detectable limit of the minor population.

Differential Flotation Centrifugation

Differential flotation was carried out according to the method described by Hijikata et al. [1993]. Fifty microliters of the sera are mixed with 8 ml of NaCl solution at a density of 1.063 g/ml and were then centrifuged in a Hitachi 70P-73 rotor at 139,500*g* for 22 hours at 14°C. After centrifugation, 1 ml of the top and bottom fractions was collected. The HCV RNA was extracted from 100 μl of each fraction, and the cDNA was synthesized as described above. The HCV cDNA was then amplified with the first PCR using the same procedures as described above. The sense primer used was 5′-TCACTCCCCTGTGAGGAACT-3′ (nt 28–47 of HCV-BK), and the antisense primer used was 5′-TGCACGGTCTACGAGACCTC-3′ (nt 311–330 of HCV-BK). The second PCR was performed using a nested primer. The sense primer used was 5′-TTCACGCAGAAAGCGTCTAG-3′ (nt 54–73 of HCV-BK), and the antisense primer used was 5′-GTTTATCCAAGAAAGGACCC-3′ (nt 179–198 of HCV-BK). One microliter of the first PCR product was mixed to the same parameters as the first PCR, and the second PCR was carried out under the same conditions as the first PCR.

In cases when the HCV RNA appeared in both fractions, the ratio of titers in the top to the bottom fractions was determined as follows: cDNA in the top and bottom fractions was diluted serially in tenfold increments, and nested PCR was then carried out. The ratio was expressed as T/B.

Additional Examinations

The HCV genotypes were determined by a two-stage PCR using mixed primers that were derived from the putative core region according to the method of Okamoto et al. [1992b]. The nomenclature for genotype was according to Simmonds et al. [1993]. The HCV RNA was quantitated by signal amplification employing branched DNA (bDNA) in a sandwich hybridization assay according to the manufacturer's instructions (Quantiplex Version 1.0; Chiron Corp.). Statistical significance of the differences was determined by Fisher's exact probability test. A P value of <0.05 was considered significant.

RESULTS

Sensitivity of SSCP

By using the first PCR products isolated from two patients, the sensitivity of SSCP was studied (Fig. 1). These products showed two bands that seemed to be a single clone (lanes 1 and 2, Fig. 1). In the SSCP analysis of a mixture of these products, the mixed bands could be seen in the same positions as the bands of lanes 1 and 2 (lanes 3–7) and were visible when diluted up to 8:05. ($=2^{-4}$ dilution; lane 7). Therefore, this SSCP analysis could detect approximately 6% of all populations.

Changes in the HVR Quasispecies and T/B Ratios Before and After IFN Therapy

SSCP analysis was carried out on the sera obtained before and immediately after the IFN therapy to examine whether the HCV populations were changed by IFN and to compare the change in the quasispecies between the patients with IR and those with NR (Figs. 2B, 3B). Nine of the twelve patients showed the changes in the quasispecies with IFN therapy, including the disappearance of the minor population. Exclusive band pattern changes were seen in 7 of the 12 patients; four of the 6 IR patients (patients 1, 4, 5, and 6) and 3 of the six NR patients (patients 7, 8, and 12) who showed almost whole band changes. There was no significant difference in frequency of the changes in the quasispecies between IR and NR patients.

Table II shows the changes in the T/B ratios before and after IFN therapy. Four of the six IR patients had a T/B ratio of 1:1, and the other two 1:10, before IFN treatment. On the other hand, all patients with NR had T/B ratios of less than 1.0. Before and after IFN therapy, the T/B ratios decreased in 6 (5 in IR, 1 in NR) of the 12 patients, was the same in 6 of the 12 patients, and increased in none.

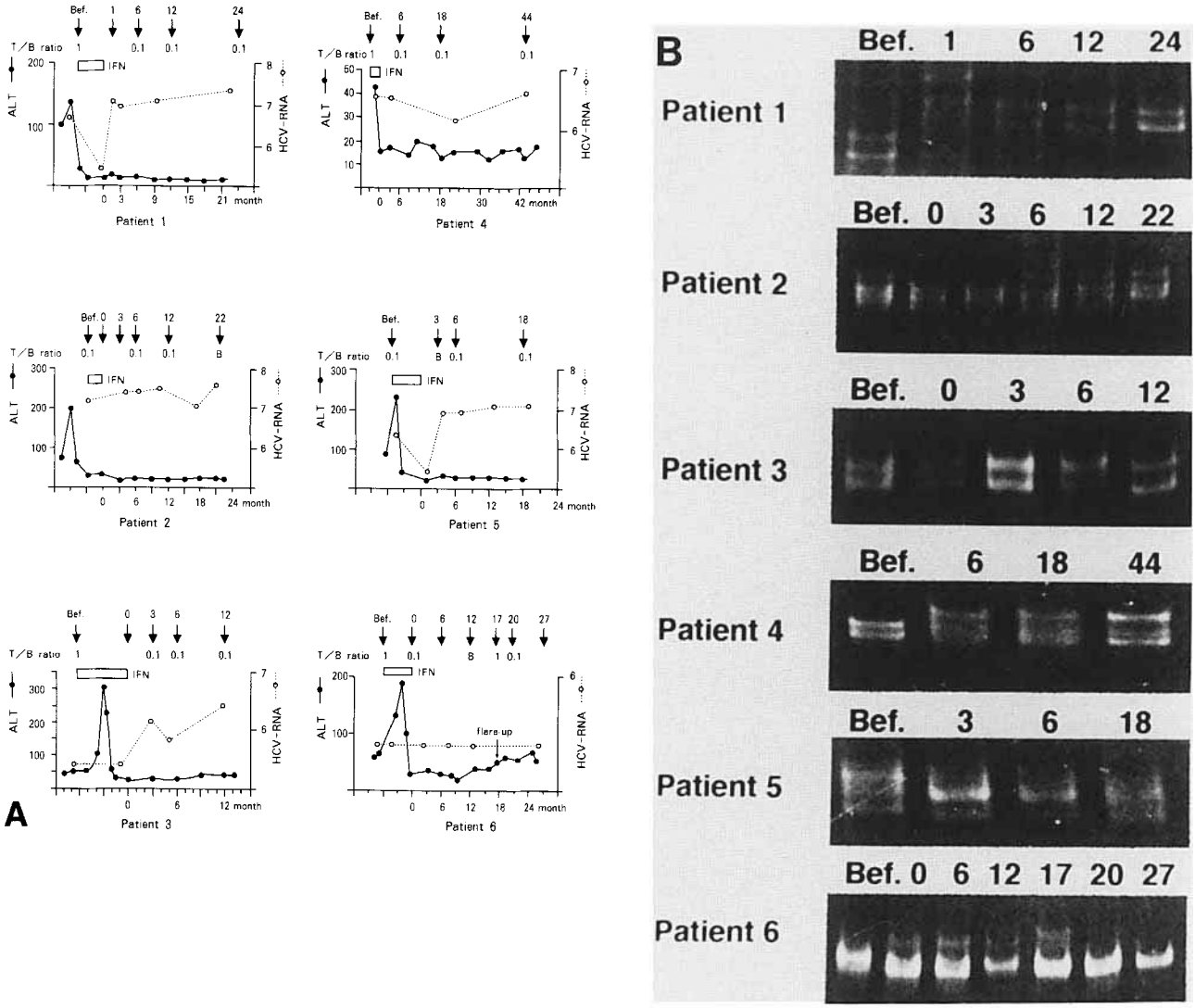

Fig. 2. Clinical courses in the IR patients and the SSCP analysis. **A:** Clinical courses. The point at the end of IFN therapy indicates time 0 (month). The vertical axis on the left side indicates ALT (IU/liter) and that on the right indicates HCV RNA levels [$\log_{10}$ (equivalents/ml)]. HCV RNA level is measured by branched DNA assay, and the detection limit of this assay is 500,000 equivalents/ml. The arrows indicate the points of SSCP analysis. Bef., before IFN therapy; T/B ratios, the ratios of HCV RNA in the top and bottom fractions (see Materials and Methods). **B:** SSCP analysis. The numbers indicate months after IFN therapy.

Changes in the HCV Quasispecies and T/B Ratios During Follow-Up After IFN Therapy

The patients were followed-up for 12–44 months (median 24 months). The periods for IR patients were 12–44 months (median 23 months) and those of NR patients 18–32 months (median 25.5 months). There were no significant differences in duration between the two response groups. Clinical features of the patients, including ALT levels, HCV RNA levels, and T/B ratios, are shown in Figures 2A and 3A. The results of the SSCP analysis are shown in Figures 2B and 3B. The SSCP bands were compared to the results at the end of therapy or at the first detectable point after therapy. Among IR patients (Fig. 2B), 3 of the 6 (patients 1, 2, and 6) showed a change in the HCV popultions. In one patient (patient 6), the ALT level rose 22 months after IFN therapy; in this patient, the bands did not change and the ALT level remained normal. Only 2 of the 6 patients (patients 1 and 2), therefore, showed a change in quasispecies despite retaining normal ALT levels. On the other hand, in all the NR patients, the different bands emerged during the follow-up period. Patients 7, 9, and 10 experienced acute deterioration of their hepatitis when their ALT levels rose to over 300 IU/liter for a short period, and HVR quasispecies changed at these points.

T/B ratios after IFN therapy were shown as 0.1 or "B" only. The T/B ratios during the follow-up were T < B in both IR and NR groups, except for patient 6 in the

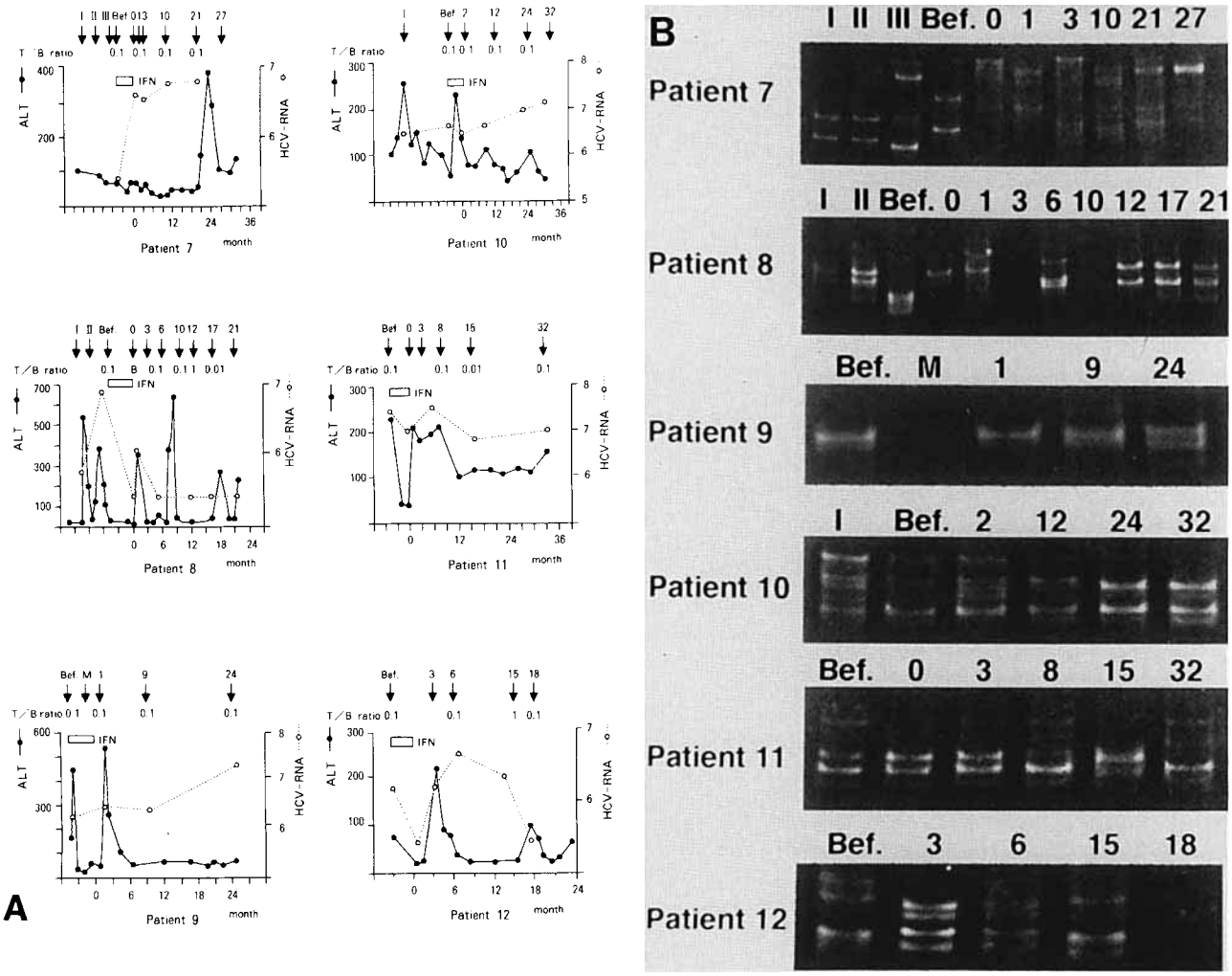

Fig. 3. Clinical course in the NR patients and the SSCP analysis. Symbols and abbreviations are explained in the legend to Figure 2. **A:** Clinical course. I, II, and III in patients 7, 8, and 10 indicate the points before interferon therapy. M in patient 9 indicates the point during interferon therapy. **B:** SSCP analysis.

TABLE II. T/B Ratios Before and After IFN Therapy

Patient[a]	T/B ratio	
	Before IFN	After IFN
IR		
1	1	0.1
2	0.1	0.1
3	1	0.1
4	1	0.1
5	0.1	B
6	1	0.1
NR		
7	0.1	0.1
8	0.1	B
9	0.1	0.1
10	0.1	0.1
11	0.1	0.1
12	0.1	0.1

[a]IR, incomplete response; NR, nonresponse.

IR group and patients 8 and 12 in the NR group (Figs. 2A, 3A). Patients 6 and 12 had normal ALT levels for at least 6 months, but the levels rose thereafter. Before the rise, the different SSCP bands emerged and the T/B ratios rose to 1.0.

DISCUSSION

Kanto et al. [1995] demonstrated that ALT levels after IFN therapy were associated with the HCV density populations. This is one possible explanation for the variation in the biochemical and virological responses among IFN-treated patients. In this study, we performed SSCP analysis and differential flotation of HCV of the patients who had undergone IFN therapy.

First, we examined the alterations in the HVR-1 quasispecies and in the T/B ratios in relation to IFN therapy. In 8 of the 12 patients studied, the pattern of the SSCP bands changed after IFN therapy; exclusively different

bands appeared in 5, minor populations disappeared in 2, and a new band appeared with the remaining bands at pretreatment in 1. These data suggest that IFN influences the HVR-1 quasispecies, although no difference was found between the IR and the NR patients in the changes of the quasispecies. On the other hand, the T/B ratios were reduced by IFN in 6 and unchanged in the remaining 6 patients. Furthermore, the patients in the IR group had a higher T/B ratio than those in the NR group. These data suggested that the virus in the top fraction might be more sensitive to IFN than that in the bottom. We had found earlier that the HCV-infected patients with T > B in the serum at pretreatment had a good response to IFN (data not shown). The virus in the top fraction is believed to be either a free virion or a lipoprotein-binding virion. The envelope structure of HCV may be one of the important factors for responding to IFN.

Second, we found that the HVR-1 quasispecies did not change or showed only a minor alteration in the IR group compared to that in the NR group. Therefore, it is thought that the ALT levels may be associated with the HVR-1 quasispecies. Furthermore, in 3 patients, the changes in quasispecies were observed before the ALT elevation, and the T/B ratio rose at the same time. This phenomenon might be explained by hepatitis due to the mutation of the HVR-1 species and the mutant increase. Hepatitis may result from a cytotoxic T lymphocyte (CTL) response against the epitope derived from viral protein [Shirai et al., 1992, 1994; Koziel et al., 1992, 1993; Battegay et al., 1995]. If HVR-1 contains the CTL epitope, the mutant in this region may result in easy CTL recognition and its attack against the liver cell infected with HCV. However, in NR patients 7, 9, 10, and 11, ALT levels were unrelated to the HVR-1 quasispecies. Therefore, it was impossible to explain all of the liver cell damage by HVR-1 alone.

In most of the patients, the T/B ratios were found to be less than 1.0 during the follow-up. Our data were not in agreement with those presented by Kanto et al. [1995] Their data showed that the T/B ratios in the IR patients were 1:10–100 and those in the other response patients were 1:1. Our data indicated that the T/B ratios did not change to less than 1.0 in the patients in whom ALT levels were approximately the same regardless of ALT levels, and the T/B ratios that were 1:1 could be seen only before the flare-up, which agrees with their findings. One of the reasons for this difference may be the methodology used. We used differentiation flotation in NaCl solution, whereas Kanto et al. performed equilibrium centrifugation in 35% sucrose. They clearly proved, using immunoprecipitation, that HCV in the top fraction was free virion and that that in the bottom was immune complex forms. Further examination into separating forms by differential flotation will be necessary.

Previous studies demonstrated that HVR-1 contains the linear neutralizing B-cell epitope and that HVR-1 quasispecies result from immune selection (that is, an escape mutant from the protective antibodies) and thus results in persistent infection. However, we must take the severity of hepatitis into consideration when investigating the relationship between the nature of the HVR-1 quasispecies and the neutralizing antibodies.

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